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Application of an integrated LC-UV-MS-NMR platform to the identification of secondary metabolites from cell cultures: benzophenanthridine alkaloids from elicited *Eschscholzia californica* (california poppy) cell cultures†

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Abstract

Plant cell and tissue cultures are a scalable and controllable alternative to whole plants for obtaining natural products of medical relevance. Cultures can be optimized for high yields of desired metabolites using rapid profiling assays such as HPLC. We describe an approach to establishing a rapid assay for profiling cell culture expression systems using a novel microscale LC-UV-MS-NMR platform, designed to acquire both MS and NMR each at their optimal sensitivity, by using nanosplitter MS from 4 mm analytical HPLC columns, and offline microdroplet NMR. The approach is demonstrated in the analysis of elicited *Eschscholzia californica* cell cultures induced with purified yeast extract to produce benzophenanthridine alkaloids. Preliminary HPLC-UV provides an overview of the changes in the production of alkaloids with time after elicitation. At the time point corresponding to the production of the most alkaloids, the integrated LC-MS-microcoil NMR platform is used for structural identification of extracted alkaloids. Eight benzophenanthridine alkaloids were identified at the sub-microgram level. This paper demonstrates the utility of the nanosplitter LC-MS/microdroplet NMR platform when establishing cell culture expression systems.

Introduction

Plant-derived natural products are a rich source of valuable medicines; examples include the painkiller morphine from the opium poppy, the anti-cancer drugs paclitaxel from the Pacific yew tree, and vincristine and vinblastine both from the *Catharanthus roseus* plant.¹ Natural products often have complex structures with multiple stereo-chemical centers, making them challenging to produce economically through chemical synthesis. Therefore, many plant-derived drugs are still supplied by extracting plant material. These compounds are produced in response to stress and pathogen attack; therefore their production is highly dependent on the season of harvest and on the environmental conditions making their production

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unpredictable. Furthermore, the supply of the desired natural products may be limited by the availability of the plant compounded by slow growth rates or over-harvesting.²

An alternative method to producing natural products is the use of plant cell and tissue cultures, which can potentially produce them at higher rates and concentrations than in whole plants. Plant cell and tissue cultures can also produce these compounds in a renewable, scalable, and reproducible way unlike harvested plants. Plant cell and tissue cultures have already been successfully applied to the large-scale and commercial production of at least fourteen compounds. Paclitaxel production yield in cell cultures is 0.5% by dry weight, compared to only 0.01% by dry weight in harvested yew trees.³

Plant cell and tissue culture systems can be manipulated to alter the metabolite profile or to enhance production of a particular compound(s) by adjusting the media or growth conditions, by selecting higher-producing cell strains, or by inducing production with biological elicitors (such as yeast extract) to mimic pathogen attack without resorting to genetic engineering.⁴ Arrays of relevant conditions can be explored in small-scale cultures before scale-up to production levels.

Optimizing metabolite production requires a rapid assay to profile the levels of metabolites in test cultures. HPLC-UV is the obvious choice for resolving the complexity of the cell cultures. It has a throughput commensurate with the typical number of test growths, tens of samples per day, and readily automated for unattended analysis. Sample preparation steps such as liquid-liquid extraction or SPE can pre-extract a family of compounds, and are parallelizable for moderate throughput, within a time comparable to setting up cultures.⁵ HPLC is also highly reproducible; the retention time of each compound is reproducible; enough to identify it relative to a standard. Where detection of one compound of interest would be minimally adequate, a full profiling of the family of metabolites would be valuable to characterize how different inducers or precursors activate and regulate different products or pathways. When coupled to a UV detector alone however, it is limited by the requirement of a chromophore for detection of compounds of interest. Additionally, in order to structurally identify compounds produced in the cell cultures, LC-UV provides limited information in the absence of an authentic standard for co-injection.⁶

To use HPLC for metabolite profiling, the peaks first need to be structurally identified, typically by mass spectrometry (MS) and/or nuclear magnetic resonance (NMR). MS provides molecular weight and, when high resolution instruments are used, the elemental composition of compounds. In addition, when used in tandem mode (MS/MS), it provides structural information from fragmentation patterns, although it is not always possible to identify structural isomers and, for unequivocal structural identification, authentic standards are required. Tandem MS can also be used to selectively screen for a particular class of compounds in a complex matrix.⁷ The high sensitivity of MS (attomole limits of detection) is not always attained due to its dependence on the ionization efficiency of some compounds or by ion suppression due to matrix effects in complex samples, although this is less of a problem when nanoelectrospray ionization is used.⁸

It is generally accepted that NMR gives the most definitive structural information. NMR provides structural information through the chemical shift and from multidimensional experiments, NMR is non-destructive, and is inherently quantitative.⁹ During optimization of cell cultures, NMR can be used to identify fingerprints of a particular class of compounds. However, NMR is limited to only the major components as NMR is inherently much less sensitive when compared to MS. Also, NMR requires relatively pure compounds thus necessitating a separation step to avoid signal overlap.¹⁰

Combining LC-MS and NMR is a powerful platform for identification of components in cell/tissue cultures as the two detectors provide complementary information to each other. Online LC-NMR (and LC-MS-NMR) methods have found some strong applications, but with on-flow NMR acquisition times under one minute, the crude spectra at the limits of detection of LC-NMR (tens of micrograms) restrict its applicability to major components of mixtures. The sensitivity of on-line LC-NMR is also limited by the volumes of LC peaks ($>100\ \mu\text{l}$) and the lower mass-sensitivity of larger NMR probes; LC peaks are usually larger than the probe active volume thus only part of the peak is sampled in NMR.¹¹

To overcome the conflicting limitations of online LC-MS and NMR, our laboratory developed a sensitive, integrated HPLC-MS-NMR platform that couples online MS with offline microcoil NMR for the analysis of mass-limited samples in complex matrices.¹² This platform provides optimal sensitivity for both MS and NMR by integrating a post-analytical column nanoSplitter with a microcoil NMR probe. By performing NMR offline from LC-MS so the entire peak volume can be concentrated into a smaller detector with >10 -fold higher mass-sensitivity and NMR acquisition may be as long as necessary to address questions remaining after review of on-line LC-MS data. The nanoSplitter provides nanoelectrospray from 4.6 mm LC columns while collecting most of the flow for offline NMR.⁸ Nanoelectrospray reduces signal suppression and increases sensitivity when compared to conventional electrospray.⁸ A microcoil NMR probe has a much higher mass-sensitivity for the limited material collected from LC because of the reduced coil diameter; noise in NMR probes is proportional to coil diameter.¹³ To facilitate the loading of small volumes of concentrated sample into a microcoil NMR probe through a large dead volume, droplet microfluidic sample loading is utilized whereby the analyte is carried in an immiscible fluid thus avoiding dispersion by confining all sample into the microcoil NMR probe's observed volume.¹⁴

In this paper, we demonstrate the applicability of our LC-MS and NMR platform to a typical case encountered when establishing new cell culture expression system; structural identification of peaks in an LC chromatogram, given one authentic standard of a compound of interest. As a model system, the method is applied to extracts of *E. californica*, elicited with a yeast extract to produce benzophenanthridine alkaloids (BPA). The effects of elicitation on *E. californica* cell cultures have been previously investigated using HPLC-UV¹⁵ and a pathway activated by elicitors was proposed in the 90's using enzymatic assays.¹⁶ The addition of an elicitor, activates the benzophenanthridine alkaloids (BPA) pathway by stimulating the expression of the Berberine Bridge Enzyme (BBE) which catalyzes the formation of (*S*)-scoulerine, the precursor for the formation of BPAs.¹⁷ The BPAs which include sanguinarine and chelerythrine are an important class of biologically active compounds which have been investigated for anti-viral, anti-inflammatory, anti-cancer, anti-microbial, and anti-fungal properties.¹⁸

The characterization of BPAs from elicited *E. californica* cell cultures has principally been done using preparative LC for conventional NMR.¹⁹ Other studies have focused on the commercially available BPAs sanguinarine and chelerythrine mainly by HPLC-UV and fluorescence studies.²⁰ Benzophenanthridine alkaloids from sources other than the *E. californica* have also been reported using mass spectrometry based methods.²¹ Due to the availability of data compiled from the literature over the last 25 years, this system was deemed suitable for evaluation of the applicability of the LC-MS-microcoil NMR platform in the characterization of natural products from elicited cell cultures at the micro-analytical scale. In this study, elicited *E. californica* cell cultures were harvested at different time-points and profiled using LC-UV. After identifying the optimal conditions which corresponded to the time-point with the most diversity, the products were characterized by

LC-MS/MS and microcoil NMR. This study required a total of 200 mg fresh weight (10 mg dry weight) of plant material.

Experimental section

Chemicals

LC-MS grade acetonitrile, water, formic acid, HPLC grade methanol and ethanol were purchased from Fisher Scientific (Pittsburgh, PA). All deuterated solvents were obtained from Cambridge Isotopes (Andover, MA). Fluorocarbon (FC-43) was purchased from 3M (St. Paul, MN). Sucrose, 2,4-dichloro-phenoxyacetic acid, L- α -naphthaleneacetic acid, sodium borohydride and the benzophenanthridine alkaloids sanguinarine and chelerythrine were purchased from Sigma-Aldrich (St. Louis, MO).

Dihydrosanguinarine and dihydrochelerythrine were synthesized from the sodium borohydride reduction of sanguinarine and chelerythrine respectively.

Maintenance and elicitation of cell culture

The *E. californica* (California poppy) suspension cultures (cell line ELDN01) were a gift from Dr Song-Yong Yoon and Dr Hwa-Young Cho of Pohang Institute of Science and Technology (POSTECH, Pohang, South Korea). Cell Cultures were maintained and elicited using the previously described method.²²

Cultures were maintained on Linsmaier–Skoog macro- and micronutrients (Caisson Laboratories, Rexburg, ID) supplemented with 30 g l⁻¹ sucrose (Sigma), 0.37 mg l⁻¹ 2,4-dichloro-phenoxyacetic acid (2,4-D, Sigma), and 0.11 mg l⁻¹ α -naphthaleneacetic acid (NAA, Sigma) and adjusted to pH 5.5 with 1 N NaOH. Sub-culturing was performed every 14 days by pipetting 20 ml of culture (containing 6 ml of packed cells) to 80 ml of fresh media. The cultures were maintained in a rotary incubator at 120 rpm, 22 °C with a 16 hour light photoperiod. Sterile water was added weekly to compensate for evaporation.

The purified yeast extract (PYE) for eliciting the cell cultures was prepared based on Hahn and Albersheim and modified as reported previously.²² Yeast extract (50 g, Becton-Dickinson, Sparks, MD) was first dissolved in 200 ml of water in a 1 L Erlenmeyer flask; then ethanol was added to a final concentration of 80% (v/v). The mixture was sealed with aluminium and parafilm stored at 4 °C for 4 days. The precipitate settled to the bottom and supernatant was discarded without filtering. The gummy precipitate was re-dissolved in 200 ml of water and precipitated again with ethanol (80% v/v) at 4 °C for 4 days. The final yeast extract precipitate was then re-suspended in 200 ml of deionized water, lyophilized for 72 hours, and stored at -20 °C. The PYE stock solution was prepared by dissolving 1 g of lyophilized PYE in 4 ml of water followed by autoclaving for 30 minutes at 121 °C.

For elicitation experiments, 2,4-D was omitted from the media to maximize alkaloid production. After growth for 7 days in to exponential phase, 25 ml of culture was transferred to a 125 ml Erlenmeyer flask and PYE was added to a final concentration of 40 mg of PYE per g fresh cell weight (FW). Cells were harvested after 24, 48, 72, and 96 hours by vacuum filtration, flash frozen in liquid nitrogen, lyophilized for 48 hours, and stored at -20 °C before for further analysis.

Alkaloid extraction

Alkaloids were extracted in 1 ml of methanol containing 0.2% (v/v) HCl as previously described.²² Cell extracts from the four time-points were first sonicated for an hour, vortexed for 30 minutes, centrifuged for 20 minutes at 13 200g at 4 °C and finally filtered through a Millex-FH 0.45 μ m syringe filter prior to HPLC analysis and LC-MS analysis.

HPLC analysis

Chromatographic separations of the 24, 48, 72 and 96 hour cell extracts were performed on an Agilent (Wilmington, DE) LC system, equipped with a binary pump, an auto-sampler, a diode array detector (Agilent 1100 series) and a fraction collector (Agilent 1200 series) controlled by Agilent ChemStation (Version B.02.01) Software. LC separations were performed on a C-18 Phenomenex (Torrance, CA) 3.5 μm , 4.6 $\times$ 150 mm, column. Separations were performed at a flow rate of 1 ml min⁻¹ with a linear gradient. The gradient was held for five minutes at 10% A (water modified with 0.1% formic acid), then increased linearly to 90% B (acetonitrile modified with 0.1% formic acid) over 90 minutes. The gradient was then held at 90% B for 5 minutes then dropped down to 10% A. A fifteen minute post-time (At 10% B) was added to allow the column to equilibrate.

LC-MS and the nanoSplitter interface

Details on the construction and design of the nanoSplitter can be found in previous publications.⁸ The nanoSplitter was used to split the flow from the LC column between the fraction collector and the MS system. 2% of the flow from the LC was directed to the MS system and 98% of the flow directed to the fraction collector. The flow directed to the nanoSplitter was then adjusted with a restriction valve so that 200 nl/minute was directed to the MS system and the rest either to waste or to the fraction collector.

MS detection was performed on a Finnigan LCQ Classic quadrupole ion trap (San Jose, CA), operated in positive ion mode. Full scan spectra between m/z 100–1000 were acquired. The source voltage was held at 2 kV and due to the low flow rates, no sheath or drying gas was required. Xcalibur software (version 1.3) was used to control the instrument and for data processing.

The instrument was tuned with sanguinarine and calibrated weekly with a mixture of Ultramark, MRFA and Caffeine (Thermo Scientific, San Jose, CA) according to the vendor's specifications.

Fraction collection

The 96 hour extract was chosen for offline high resolution MS and NMR analysis. The LC eluent was split with a restriction valve and 98% of the flow was directed to the fraction collector *via* the diode array detector (the delay volume between the UV and the fraction collector was 71 μl). The fraction collector was operated in a time-dependent mode at five fractions per minute. The fractions were collected in low retention 96-well PCR plates (Nunc, Rochester, NY) with 200 μl deposited in each well. Wells containing the same peak of interest were pooled together into Eppendorf tubes for high resolution MS and NMR analysis. To reduce contamination from plasticizer phthalates, the PCR plates and Eppendorf tubes were soaked for an hour in a tub filled with 100% methanol before use.

High resolution MS

For high resolution mass spectrometry, 10 μg of the 96 hour extract was injected on the LC column and fractions of the peaks of interest collected as described above. Wells containing the same peak of interest were pooled, and then infused directly without drying into an LTQ-Orbitrap XP (Thermo Fischer, San Jose, CA) at a flow rate of 10 μl min⁻¹. The mass spectrometer was operated in the positive ion mode with the source voltage and heated capillary temperature set to 4.5 kV and 275 °C respectively. Nitrogen was used as a sheath gas.

Full MS and MS/MS spectra of between m/z 100–500 were acquired for each peak (1–8), at 60k resolution, with 3 micro-scans. MS/MS analysis, collision induced dissociation (CID) of

the peak of the most intense ion with was performed in the LTQ, and the fragments ions measured in the Orbitrap with an isolation window of 1 m/z . Fragmentation was activated for 0.25 ms, normalized collision energy of between 30 and 40% was used for each analyte. Xcalibur software (Version 2.1) was used to control the instrument and for data analysis. The fragmentation method was optimized using direct infusion of a solution of sanguinarine standard at a flow rate of 10 $\mu\text{l min}^{-1}$.

Mass calibration was performed according to the method provided by the instrument vendor. The mass accuracy of all fractions of interest was below 3 ppm.

NMR analysis

NMR analysis was performed on an Inverse Carbon Gradient (ICG) probe (MRM/Protasis, Savoy, IL). This probe has an observed volume of 2.5 μl . The probe was internally coated with fluoro-octosilane for use with microdroplet NMR as previously described.¹⁴

All NMR spectra, were acquired on a Varian (Palo Alto, CA) Inova spectrometer with an 11.7-T (500 MHz) actively shielded magnet; the data were processed and analyzed with VNMR version 6.1C software. 1k to 16k scans were acquired for each fraction depending on the concentration of each sample. Spectra were acquired with a 60 degree tip angle, 1.98 s acquisition times, and when necessary, pre-saturation of the residual water peak using a saturation delay of 1.5 s was performed. All spectra were collected at a fixed gain of 60 (maximum gain). The spectral width was set to 8 KHz. Each FID was zero-filled to 64k points, multiplied with an exponential function (1 Hz line broadening), phased manually, and, baseline corrected. Chemical shifts were referenced to deuterated DSS.

Before NMR analysis, the LC solvent of the pooled fractions was dried under vacuum and the fractions re-suspended in 5 μl deuterated DMSO. The samples were loaded into the probe as immiscible droplets of water in oil as follows: a 25 μl Hamiltonian syringe fitted with 10 cm Teflon tubing (200 μm I.D.) with a 4 cm 200 μm capillary stub was filled with the immiscible carrier fluid FC-43 to the 15 μl mark, 3 μl of the sample was then drawn into the syringe, followed by 2 μl of FC-43 and finally deuterated DMSO as a wash plug. The sample was pumped into the probe with a syringe pump flowing at 1 $\mu\text{l min}^{-1}$. The centering of the sample in the probe's active volume was determined by the maximum lock.

Compounds **2** and **3**, which showed the presence of more than one co-eluting compound in NMR, were recovered after NMR analysis to ensure they had not degraded during NMR analysis. Each compound was recovered by back-flushing the microcoil probe with 25 μl of FC-43 (total probe dead volume is 17 μl). Each recovered fraction contained a layer of immiscible fluoro-carbon (bottom layer) and sample. The sample layer (top layer) was pipetted out and dried down under vacuum. Each recovered fraction was then re-suspended in starting mobile phase (10% acetonitrile/90% water) then re-injected into the LC-MS system using the same method used for separation of the cell extracts.

Results and discussion

An overview of the experimental design used in this work is illustrated in Fig. 1. An extraction and LC method was developed for the *E. californica* cell cultures, and several growth conditions were examined (time-points after elicitation), prior to requesting the expense and expertise of structural assignment of the chromatogram. A sample was selected with the best representation of peaks of interest at sufficient level for identification, and analyzed with the nanoSplitter LC-MS using a unit resolution MS (ion-trap), while collecting time-based fractions. After review of the LC-MS data, fractions were pooled to recover single-compound peak. Each peak was infused into a high resolution MS (LTQ-

Orbitrap XP) to determine the exact mass and to obtain tandem MS fragmentation (MS/MS). Fractions for NMR were concentrated by drying off the LC solvent, re-suspended in deuterated dimethyl sulfoxide (DMSO-d₆) then loaded into the microcoil NMR probe using droplet micro-fluidics (more details in methods). The MS and NMR data from the micro-analytical platform were then interpreted with reference to analysis of one standard, sanguinarine (SA). The results were confirmed by comparison with the previously published (large-scale) studies of *E. californica*.¹⁹ The benzophenanthridine alkaloid (BPA) chelerythrine was also used for confirmatory purposes of one of the isolated compounds. Eight compounds were isolated, down to the 200 ng level, using the nanoSplitter LC-MS/Microdroplet NMR platform from analytical scale LC separation loading of 50 µg of extract.

LC-UV profiling of elicited cell cultures

Fig. 2 shows a time-course of alkaloid production after elicitation, monitored by LC-UV. An overall pattern of five early eluting peaks (1–5) and three late eluting peaks (6–8) is evident. This pattern reflects the two forms of BPAs: the more polar quaternary benzophenanthridine alkaloids (QBPA), represented by peaks 1–5, and the non-polar dihydrobenzophenanthridine alkaloids (DHBPA), peaks 6–8 which elute late under reversed phase LC conditions.¹⁶ The total amount of all the BPAs combined, accumulated roughly linearly from 7 mg g⁻¹ dry weight (DW) at 24 hours after elicitation, to 20 mg g⁻¹ DW at 72 hours as illustrated in Fig. 2b. The compounds in peaks 6–8 were observed 24 hours after elicitation and their accumulation remained relatively constant between 24 and 96 hours after elicitation, while the accumulation of the compounds in peaks 1–5 increased with time, with the compounds in peaks 2 and 5 not accumulating to a detectable level until 72 and 48 hours respectively, after elicitation. Longer harvest times are therefore preferred for accumulation of peaks 1–5. Compounds corresponding to Peaks 6–8 can be harvested at any time point. In order to identify the compounds produced from a single chromatogram after elicitation, the 96 hour extract was selected for LC-UV-MS and NMR analysis because it presented the most balanced presence of all the peaks in the mixture.

Identification of LC peaks

After initial LC-UV analysis had established that the 96 hour extract presented the most balanced profile of all of the compounds in the elicited cell culture, this time-point was selected for more detailed characterization of the individual compounds by LC-MS-microcoil NMR. Given the high mass-sensitivity of the microcoil NMR (*ca.* 200 ng as compared to *ca.* 10 µg for conventional 5 mm NMR probes) isolation of compounds of interest could be conducted on the 4.6 mm columns as opposed to semi-preparative columns.¹³ To prevent column overload that led to peak-to-peak contamination from peak broadening, the total amount of extract loaded on the LC column for any given injection was kept under a maximum of 50 µg. In addition, three repeat injections were pooled to increase the amount of material collected for NMR analysis of the smaller peaks (note that tripling the amount of material for NMR reduces the time requirement for NMR acquisition by nearly 10-fold). Repeating an LC collection only required an additional two hours per run and could be done overnight under LC automation, which took considerably less time when compared to NMR analysis time. Fractions that contained the same chromatographic peak from the different LC runs were then pooled manually for microcoil NMR analysis. The mass of each peak collected was estimated by UV, to aid in allocation of the NMR acquisition time to obtain an acceptable (>10) signal-to-noise ratio. For example, the NMR spectrum for Peak 4 (8 µg) was acquired in an hour, while the spectrum of Peak 2 (200 ng) required an overnight acquisition.

The LC-UV-MS chromatograms and ^1H NMR spectra of the 96 hour extract are shown in Fig. 3. All the peaks detected by UV were also observed in the MS chromatogram, although Peak 6 gave a strong signal in UV but a small peak in MS, presumably due to low ionization efficiency. The correlation of the UV and MS signals was important because the pooling of fractions for offline analysis (exact mass and NMR) was based on the MS response while fraction collection was correlated to the UV. At unit mass resolution, some peaks exhibited the same nominal mass *e.g.* compounds 2 and 3 (Table 1). All the LC peaks above a 0.1% threshold in the UV chromatograms, were targeted for offline high resolution MS and NMR analysis. High resolution MS was used to determine elemental composition and for MS/MS analysis as a first step towards structural assignment of the BPAs. MS/MS showed various similar neutral losses from the peaks of the chromatogram an indication that the isolated compounds were related. Exact mass MS/MS results also established the elemental composition of the fragment ions, pinpointing the chemical moiety present in the BPAs as summarized in Table 1. The BPAs have the same core structure and differ only in functional group and substitution patterns around the four fused rings. NMR was used to determine the site of modification and to confirm MS results; spectral differences were mainly in the aromatic region (Fig. 3B).

MS and NMR features of sanguinarine (SA)

When establishing a cell culture expression system, there is usually at-least one well characterized compound of interest. The MS and NMR data (Fig. 4) of the standard Sanguinarine (SA) were used as a reference to assist in the interpretation of the MS and NMR data of the LC-UV peaks in the 96 hour cell extract. Because the ESI mass spectrum was obtained from an acidic solution which put SA into the positively charged quaternary nitrogen form, its peak of highest mass corresponded to the actual molecular mass, M^+ , and occurred at an even mass, m/z 332, even though SA contains a single nitrogen atom. The MS/MS spectrum of SA exhibited fragment ions at m/z 317 $[\text{M} - \text{CH}_3]^+$, m/z 304 $[\text{M} - \text{CO}]^+$ and at m/z 274 $[\text{M} - (\text{CH}_2\text{O} + \text{CO})]^+$. Although the acidic form of SA was observed under positive electrospray conditions, in the ^1H NMR analysis of the LC-fractionated SA, the neutral form (pseudo-base) was observed (Fig. 4C). This is in accord with the previously published results showing that QBPA's like SA exist in two forms, which are dependent on the pH of the solution^{7,23} (Fig. 4B). The aromatic region of the ^1H spectrum of SA-pseudo-base (Fig. 4C) exhibited six protons, two as singlets and four as ortho-coupled doublets ($J = 8.8$ Hz). In addition, the ^1H spectrum exhibited a methyl singlet at 2.8 ppm from the N- CH_3 group and resonances between 6.1 and 6.15 ppm from the methylenedioxy groups at C-2 and C-7. In the subsequent evaluation of the NMR spectra of Peaks 1–8 discussed below, the NMR resonances of the basic form of SA are used as a reference.

The first LC peak, 1 (m/z 332, $[\text{M}]^+$) was assigned as SA on the basis of the comparison of its MS and ^1H NMR spectra with a reference sample of SA. In addition under the same chromatographic conditions, its retention time was the same as that of SA. Furthermore, co-injection with sanguinarine, yielded a single peak.

The second and the third LC peaks, 2 and 3 exhibited the same nominal masses (m/z 348, $[\text{M}]^+$), but different exact mass (elemental composition) and MS/MS spectra, as summarized in Table 1. The MS/MS spectrum of compound 2 exhibited neutral losses similar to SA with fragment ions at m/z 333 $[\text{M} - \text{CH}_3]^+$, m/z 320 $[\text{M} - \text{CO}]^+$, and m/z 290 $[\text{M} - (\text{CH}_2\text{O} + \text{CO})]^+$, an indication that the two compounds were structurally related. Its exact mass suggested that a hydroxyl group had been added to SA. The MS/MS spectrum of peak 3 on the other hand, exhibited fragment ions at m/z 333 $[\text{M} - \text{CH}_3]^+$, m/z 318 $[\text{M} - 2\text{CH}_3]^+$, 315 $[\text{M} - (2\text{CH}_3 + 2\text{H})]^+$, m/z 304 $[\text{M} - (\text{CO} + \text{CH}_3 + \text{H})]^+$.

To identify the differences and similarities of **2** and **3** to SA, their ^1H spectra were acquired. However, although the two compounds exhibited individual peaks in LC, their ^1H NMR spectra (Fig. 3B) suggested that the chromatographically isolated peaks contained more than one co-eluting species. To ensure that the extra signals observed in ^1H NMR were due to co-eluting species and not from degradation, after ^1H NMR acquisition each of the two compounds was recovered and re-injected into the LC-MS system, each eluted as a single peak at the retention time observed during fractionation. The two compounds were therefore tentatively identified by their MS and MS/MS results and in the case of compound **3** by comparison with the BPA chelerythrine (CHE) which is commercially available. Co-injection of **3** with CHE yielded a single peak and the MS and MS/MS spectra of **3** and CHE were identical. Compound **2** was tentatively identified as 10-hydrosanguinarine based on its exact mass and MS/MS spectra.

The molecular mass of the fourth LC peak, **4** (m/z 362, $[\text{M}]^+$) was 30 mass units above that of SA, consistent with the addition of a methoxy group to SA, as also confirmed by exact mass measurements. The MS/MS spectrum of **4** exhibited similar neutral losses as sanguinarine, of $[\text{M} - \text{CH}_3]^+$ and $[\text{M} - \text{CO}]^+$, an indication of some structural similarity to SA. The presence of the methoxy group was supported by the presence of a three-proton singlet at ~ 3.9 ppm in its ^1H spectrum. To determine the site of the methoxy substitution the ^1H NMR spectra of **4** and SA were compared. The presence of five protons (two ortho-coupled doublets and three singlets) is evident in the aromatic region of the ^1H NMR spectrum of **4**. This compares with six protons in SA, seen as four ortho-coupled doublets and two singlets. Specifically, in SA, H-9 is an ortho-coupled doublet but is found as a singlet in **4**, and is shifted upfield to 6.98 ppm from 7.02 ppm in SA. In addition, H-11, which is the most de-shielded proton in SA is shifted downfield to 8.34 ppm in **4** from 7.83 ppm in SA, and this can be attributed to the through-space interactions of that proton with the methoxy group. Compound **4** can thus be identified as chelirubine. Our assignment of compound **4** as chelirubine is in agreement with previously published results.²³

The mass spectrum of LC peak **5**, with a molecular mass (m/z 392, $[\text{M}]^+$) which is 60 mass units above that of SA, suggested the addition of two methoxy groups to SA. The MS/MS spectrum of **5** also exhibited the same neutral losses (Table 1) as SA and analytes **2** and **4**, an indication that they shared common functional groups. The addition of the two methoxy groups to **5** was confirmed by its ^1H spectrum which showed methoxy singlets at 3.7 and 3.9 ppm. The aromatic region of the ^1H NMR spectrum of **5** exhibited four one-proton singlets; the two singlets at 7.4 ppm (H-1) and 7.6 ppm (H-4) which were also observed in SA; H-9 and H-11 were singlets in **5**, but were doublets in SA, an indication that C-10 and C-12 were the sites of substitution. This conclusion is further supported by the absence of the doublets from H-10 and H-12 seen in SA. The pattern of resonances in the aromatic region of compound **5**, matches previously published ^1H results of marcapine thus **5** is assigned as marcapine.¹⁹

Characterization of late eluting LC Peaks 6–8

In comparing the MS, MS/MS and ^1H NMR features of the early eluting compounds, **1–5**, and the late eluting cluster, **6–8**, several distinct features as well as similarities became apparent. Some key difference common between peaks in the two clusters of compounds is observed in their respective ^1H NMR spectra as illustrated in Fig. 5, which shows an expansion from the ^1H NMR spectra of compounds **4** and **7**. Although the aromatic regions of the NMR spectra are similar, a methine proton signal is observed at ~ 5.3 ppm (H-6) in the spectrum of **4**. This signal is shifted upfield to ~ 4.05 ppm and integrates to two protons, reflecting the presence of a methylene group. A similar shift of H-6 is observed in all the other late-eluting peaks. This structural change is also reflected in the mass spectral results.

While both sets of compounds exhibited similar neutral loss fragments in their MS/MS spectra, the MS peak representative of the molecular mass of each compound differed in that while the highest mass ion in the spectra of **1–5** corresponded to $[M]^+$, the ESI spectra of the late eluting peaks produced the protonated adduct ($[M + H]^+$). As discussed earlier for SA, this is because the early eluting peaks (QBPA)s are already charged in solution while the late eluting peaks (DHBPAs) elute in their neutral form. These general features were observed in the analysis of **6–8** and along with data from the reference sample of SA were used in their structural characterization as discussed below.

The NMR spectrum of the sixth LC peak **6** (m/z 350, $[M + H]^+$) exhibited a number of similarities to that of sanguinarine in its aromatic region where it shared the same six protons, four as ortho-coupled doublets ($J \approx 8$ Hz) and two as singlets, but differed by the additional presence of two methoxy singlets at ~ 3.7 ppm and at ~ 3.9 ppm. In addition, unlike SA which had two methylenedioxy groups, compound **6** had one methylenedioxy group as shown by the singlet at ~ 6.1 ppm. Accordingly, **6** was assigned the structure of dihydrochelerythrine. This assignment was further supported by the reduction of the commercially available standard chelerythrine with NaBH_4 to its dihydro derivative, and when the dihydro derivative was analyzed by MS and NMR, its MS and NMR results matched those of **6**. Also, LC co-injection of isolated **6** and dihydrochelerythrine yielded a single peak.

As discussed above, the ^1H NMR spectra of compounds **7** (m/z 364, $[M + H]^+$) and **4**, both exhibited five protons in the aromatic region, two ortho-coupled doublets and three singlets. **7** also exhibited a methoxy singlet at ~ 3.9 ppm. The main difference in their ^1H spectra was in the two proton singlet at ~ 4.1 ppm in **7**, which appeared as a one proton singlet in **4**, indicating that **7** had two protons at C-6 unlike **4** which had one proton. Peak **7** was therefore assigned as the structure of dihydrochelirubine, the dihydro derivative of **4**. This was further confirmed by the NaBH_4 reduction of **4** to its dihydro derivative and comparison the ^1H NMR results with those of **7**.

Finally, the ^1H NMR spectrum of the eighth LC peak, **8** (m/z 334, $[M + H]^+$) exhibited the same pattern of resonances in its aromatic region as SA, with six protons in its aromatic region, two as singlets and four as ortho-coupled doublets. Its ^1H spectrum differed from that of SA, in that the methine resonance at 5.5 ppm in SA was shifted upfield to 4.1 ppm and was indicative of a two-proton singlet. Compound **8** was therefore identified as dihydrosanguinarine and the assignment is further supported by the NaBH_4 reduction of sanguinarine to its dihydro derivative. The MS and the ^1H NMR spectra of the reduced SA derivative, matched the results from compound **8**.

In summary, the complementary use of MS and NMR identified six of the eight compounds in the 96 hour extract, and exact mass measurements aided in differentiating compounds of the same nominal mass (compounds **2** and **3**). Crucial for the complete structural characterization of the compounds in the cell culture was the availability of a reference compound in this case sanguinarine, which contained the core structure of most of the likely products. The MS/MS data brought out the similarities of the profiled compounds as they all originate from dihydrosanguinarine, with the exception of chelerythrine and dihydrochelerythrine which are derived directly from *S*-scoulerine (Fig. 6). The ^1H NMR spectrum of sanguinarine provided a reference for identification of the exact site(s) of modification of the core structure in most of the isolated metabolites, thus removing the requirement for more time consuming 2D-NMR experiments. In principle, after their full characterization, the isolated compounds can be used as standards in other studies either for method development or in analysis of cell cultures producing related compounds. The

results from our microanalytical platform are consistent with data obtained from previous studies carried out at the preparative scale using 400-fold larger amounts of sample.¹⁹

As shown in Fig. 6, *E. californica* cell cultures produce twelve compounds compared to our detection of eight in the cell cultures employed in the present work. It is conceivable that the remaining four could either be at low levels, below the detection limit of our analytical platform or these compounds do not accumulate under the specific conditions employed in our cell cultures. Additionally, the ¹H NMR spectra of **2** and **3** showed the presence of more than one co-eluting compounds. It is possible to improve the selectivity of our LC method to resolve the other compounds in the two peaks either by changing the mobile phase or by changing the column chemistry. It should be noted however that changing selectivity in LC might change the elution order and selectivity of the other peaks detected by our method.

Conclusion

The data presented above demonstrate the applicability of a novel integrated LC-UV-MS-microNMR platform for the identification of compounds from a plant cell culture at the analytical scale. The platform has a large dynamic range as demonstrated in the analysis of the *E. californica* cell cultures, where structure characterization was carried out in elicited cell cultures containing metabolites ranging from as little as 200 ng to as high as 8 µg. With these identifications, LC-UV serves as a rapid method for profiling metabolites produced in cell cultures under different elicitation and growth conditions. For final structure characterization, this analytical approach allows for the use of each detector at its optimal sensitivity by having NMR offline from MS, so each method is applied independently without compromising its performance. Having LC-MS offline from NMR also separates the expertise required into the established fields of LC-MS and NMR, obviating the requirement for an expert team on hyphenated LC-MS-NMR. The simultaneous use of MS and UV as a guide for detection and collection of peaks of interest for offline NMR analysis is advantageous because it can be extended to the analysis of LC peaks that can be monitored by either detector regardless of whether they contain a UV chromophore or not. The non-destructive nature of NMR is valuable in that compounds of interest can be recovered for re-injection as demonstrated with compounds **2** and **3** or for further analysis on other analytical platforms.

While the focus of this publication was in demonstrating the utility of the LC-UV-MS-offline microNMR analytical platform in the identification of the compounds in the *E. californica* cell culture, it should be noted that its general applicability extends to a broader spectrum of preliminary screening and optimization of bioengineered cultures and, indeed, to identification of natural products or drug metabolites in general. We have illustrated LC-MS + NMR using our platform of concentrating collected fractions by drying for later analysis by microcoil NMR, but these same advantages can be gained with other forms of fraction collection, such as SPE cartridges,²⁴ followed by offline NMR analysis using any of a variety of highly sensitive NMR probes available, such as micro-cryoprobes with 1 mm or 1.7 mm tubes.²⁵

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

The work-flow used in identification of secondary metabolites in cell cultures. After extraction of the metabolites, an LC method is developed that resolves the complexity of the mixture. Different cell culture conditions are surveyed by LC-UV.

Fig. 2. Accumulation of Benzophenanthridine alkaloids (BPA) with time: (A) LC-UV (283 nm) chromatograms showing a time-course of detected metabolites. The 96 hour extract is further analysed by LC-MS and NMR. (B) The bar graph represents the total accumulation of indicated peaks in (A) over time (done in triplicate). The total BPAs accumulate roughly linearly with time from 24 to 96 hours after elicitation.

Fig. 3.
(A) LC-UV-MS-NMR of the 96 hour time-point *E. californica* extract: (TOP) UV (283 nm) and MS chromatograms correlate to within 0.1 minutes (MS chromatogram is shifted to the right for clarity). The LC peaks labeled **1–8** were fractionated for offline high resolution MS, MS/MS and, microcoil NMR analysis. (B) Microcoil ¹H NMR spectra of the aromatic region (6.5 to 9 ppm) of the LC-UV Peaks **1–8**. The mass of collected analytes varied from 200 ng (**2**) to 8 μ g (**4**).

Fig. 4. (A) MS/MS spectrum of Sanguinarine (SA). (B) The conversion of quaternary benzophenanthridine alkaloids to the pseudo-base from the acidic form (left) in LC-MS to the pseudo-base neutral form (right) in neutral solution. (C) ^1H NMR spectrum (δ 5–9 (left), δ 2.0–3.0 (right)) of sanguinarine pseudo-base, with NMR peak assignments.

Fig. 5.

^1H NMR spectra (δ 3.8–6.6) of compounds **4** (top) and **7** (bottom) representative of the common difference between the early eluting compounds (**1–5**) and the late eluting compounds (**6–8**). The singlet (C-6, one proton) at δ 5.3 in Peak 4, is shifted up-field to δ 4.1 (C-6, two protons) in **7**. The early eluting compounds are the quaternary benzophenanthridine alkaloids which are converted to their dihydro derivatives (**6–8**) by the enzyme dihydrobenzophenanthridine alkaloid oxidase. Compound **7** is the dihydro derivative of compound **4**. The neutral (pseudo-base) form of **4** shown in the figure is the form in deuterated DMSO for NMR.

Fig. 6. The benzophenanthridine alkaloids pathway that is activated when *E. californica* cell cultures are elicited (adapted from ref. 16).

Table 1

The nominal mass, exact mass and major fragments ions of compounds **1–8**. The exact mass error was below 5 ppm for all the peaks

	Observed nominal mass	Observed exact mass	Major fragment ions	Calculated exact mass	
Peak 1	332	332.0909 [M] ⁺	317.0680, 304.0964, 274.0861	332.0912	Sanguinarine
Peak 2	348	348.0858 [M] ⁺	333.0626, 320.0910, 290.0808	348.0861	10-Hydrosanguinarine ^a
Peak 3	348	348.1216 [M] ⁺	333.0982, 315.0890, 304.0960	348.1225	Chelerythrine ^a
Peak 4	362	362.1015 [M] ⁺	347.0783, 334.1074	362.1018	Chelirubine
Peak 5	392	392.1123 [M] ⁺	377.1130, 364.1353	392.1123	Marcapine
Peak 6	350	350.1374 [M + H] ⁺	335.1140, 319.1193	350.1387	Dihydrochelerythrine
Peak 7	364	364.1165 [M + H] ⁺	349.0920, 333.0987	364.1179	Dihydrochelirubine
Peak 8	334	334.1060 [M + H] ⁺	319.0826, 304.0960, 276.1013	334.1074	Dihydrosanguinarine

^a Compounds **2** and **3** were tentatively identified as 10-hydrosanguinarine and chelerythrine based on their MS and MS/MS results.